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SHORT COMMUNICATION

DECREASED EXPRESSION OF THE MITOCHONDRIAL BCAT PROTEIN CORRELATES WITH IMPROVED PATIENT SURVIVAL IN IDH-WT GLIOMAS

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Abstract

Background and research question: Gliomas represent 43% of all solid intracranial tumours, of which glioblastomas have the poorest prognosis. Recently, the human cytosolic branched-chain aminotransferase protein (hBCATc), which metabolises the branched-chain amino acids (BCAA), was identified as a biomarker and therapeutic target for glioblastomas carrying wild-type isocitrate dehydrogenase (IDH-WT) genes. However, the clinical utility of the mitochondrial isoform, hBCATm, which also metabolises BCAAs, was not determined nor its potential role in predicting patient survival.

Methods: Glioblastomas, of grades II-IV, from 53 patients were graded by a neuropathologist, where the IDH and MGMT status were assessed. Tumours positive for hBCATm, hBCATc and BCKDC were characterised using immunohistochemistry and Western blot analysis using antibodies specific to these proteins.

Results: Here, we report that in IDH-WT tumours, the expression of hBCATm is significantly increased (*p=0.034*) relative to IDH mutation gliomas, and significantly correlates with patient survival, on Kaplan-Meier analysis, where low hBCATm expression is a positive prognostic factor (*p=0.003*). Moreover, increased hBCATm expression in these glioblastomas correlated with tumour grade indicating their role as a predictive biomarker of glioma progression. Multiple banding was observed for the branched-chain α-keto acid dehydrogenase complex, which catalyses the committed step in BCAA metabolism, but a significant change in expression was absent (*p=0.690*).

Conclusion: Until now, IDH-WT glioblastomas have a uniformly poor prognosis, however we demonstrate for the first time that relatively low hBCATm may select for a better performing subset within this group and may represent a therapeutic target in these hard to treat patients.

Keywords: Glioblastomas, therapeutic target, biomarkers, branched-chain aminotransferase, hBCATm, hBCATc

Introduction

Although histologically indistinguishable, glioblastomas vary in their clinical, genetic and epigenetic profiles (Ohgaki and Kleihues, 2013;Kreth *et al*., 2014). Primary glioblastomas, which arise de novo, may show epidermal growth factor receptor (EGFR) amplification and PTEN mutations, compared with secondary glioblastomas that progress from lower grade astrocytomas, which occur in younger patients, and typically carry mutations including isocitrate dehydrogenase IDH1/2, which tend to be absent in primary glioblastomas (<5%) (Balss *et al*., 2008;Guo *et al*., 2011). Although glioblastomas with IDH mutations are associated with an improved prognosis and a hypermethylated phenotype, hypermethylation of the O^6-methylguanine-DNA methytransferase (MGMT) gene promoter can also be seen in primary glioblastomas in the absence of IDH mutations (Guo *et al*., 2011;Esteller *et al*., 2000;Esteller *et al*., 2000). Conversely, IDH-wild-type (IDH-WT) tumours that exhibit an increased expression of the cytosolic human branched-chain aminotransferase protein (hBCATc) are linked with a poor prognosis (Tönjes *et al*., 2013). Tönjes and co-authors showed that increased expression of hBCATc supports tumour growth and highlighted it as a new therapeutic target for glioblastomas.

The hBCATc protein is however one of two isoforms (the other, mitochondrial (hBCATm)) that can catalyse the transamination of the branched-chain amino acids (BCAA) followed by complete oxidation by the branched-chain α-keto acid dehydrogenase complex (BCKDC) (Conway and Hutson, 2015). Like BCAT1 (gene encoding hBCATc), a significant up regulation of BCAT2 (gene encoding hBCATm) was reported in IDH-WT tumours (Tönjes *et al*., 2013). However, hBCATm expression was not studied at the protein level nor assessed as a prognostic marker for patient survival. It is important to consider hBCATm expression and indeed the expression of the BCKDC in IDH-WT tumours because their existence will also contribute to BCAA metabolism promoting tumour survival. This is of relevance because, if hBCATc was considered the only contributor to BCAA metabolism and thus the sole therapeutic target, then if hBCATm expression was found to be increased in

glioblastomas it could potentially attenuate a hBCATc inhibitor. Therefore, in light of this and other studies where expression of hBCATm is raised under proliferative conditions (Pérez-Villaseñor *et al*., 2005) and our studies which show that hBCATm expression, more so than hBCATc, is significantly increased and correlates with Braak stage in Alzheimer's disease (Hull *et al*., 2015;Hull *et al*.,2015) we examined the differential expression of hBCATm and BCKDC in IDH-WT and IDH mutation glioblastomas and determined if their expression correlated with patient survival. Our interest also extends to the potential for these metabolic proteins to show value as biomarkers between tumour grades, offering increased clinical utility for these proteins.

Materials and methods

Patient Samples

The study was approved by the Brain Tumour Bank South West Research Ethics Committee, Bristol, UK. For Western blot analysis, 64 glioma tumours with a range of sub-types were compared for BCKDC, hBCATc, and hBCATm expression (Supplementary Table 1). For IHC 53 glioma cases were graded by Dr. Kurian, consultant neuropathologist at Southmead Hospital Neuropathology Department, Bristol, UK. All glioma cases had a diagnosis from grade II to IV.

IDH and MGMT analysis

DNA was extracted from fresh, frozen tissue using the BioRobot EZ1 workstation (Qiagen). Sanger sequencing of the p.Arg132 region of IDH1 (codons 55 – 138) and p.Arg172 region of IDH2 (codons 151 – 179) was completed using a 3730 DNA analyser (Applied Bio-Systems). Immunohistochemistry (IHC) was performed using standard techniques with the antibody clone H09 (Dianova), which specifically reacts with the isocitrate dehydrogenase 1 (IDH1) R132H point mutation (1:10 dilution for 30 min at room temperature) in formalin-fixed tissue. MGMT analysis was assessed by methylation sensitive PCR. DNA samples underwent bisulphite modification, followed by PCR using the primers described by Estellier *et al* 2000 in a diplex reaction. PCR products were separated on the Beckman Coulter CEQ 8000 system.

Immunohistochemistry

Sections (3 µm in thickness) were taken onto 3-aminopropyltriethoxysilane coated adhesive slides and prepared for incubation with antibodies as previously described.[10,12] Primary antibodies included: anti-hBCATm, 1:1000; anti-hBCATc, 1:6000; anti-BCKDC (ab126173, raised to the E1α subunit), 1:6000, incubated overnight (~20 hours) at room temperature. Standard IHC was performed as per Vectastain ABC and DAB substrate protocol, prior to immersion in copper sulphate (16 mM $CuSO_4.5H_2O$, 123 mM NaCl) for 4 minutes and

counterstained with Harris's haematoxylin (25% Gill haematoxylin). Sections were viewed, scored and imaged on a Nikon Eclipse 50i and 80i microscope.

Scoring protocol

Each image was scored based on the degree of DAB staining. The sections were examined under a 20X objective and the degree of labelling designated on a semi-quantitative scale: 0 = no staining, 1 = mild, 2 = moderate and 3 = intense staining. Scoring of each slide was independently performed by 3 individuals, with the median score used to obtain consensus. The analysis was conducted blind to mutation status, grade and age at diagnosis of each patient. The corresponding area of tissue was found with each of the antibodies in order to allow direct comparison of tumour cells. Negative antibody controls, where primary antibody was replaced with PBS, were supplied by Frenchay Neuropathology Department.

Western blot analysis

For Western blot analysis, 250 mg of tissue was collected in RNase free tubes and homogenised as described in Hull *et al.*, 2015a. Primary antibody (anti-BCKDC, 1/5000; anti-hBCATc, 1/5000; anti-hBCATm, 1/5000; anti-tubulin 1/000, 1/10 000 anti-GAPDH, 1/10 000) was prepared in 5% non-fat milk powder in TBST and incubated overnight (20 hours, 4°C) prior to TBST washes. Secondary antibody (1/5000) linked with HRP, was added for 1 hour and then washed with TBST. The positive bands were visualized using chemiluminescent HRP substrate. GBX developer and fixer were used for 40 and 20 seconds respectively. Integrated area densitometry was carried out with ImageJ. For re-probing, membranes were incubated with 1 M NaOH (7 minutes) and washed in TBST.

Statistical analysis

Data validity checks and descriptive analyses were performed prior to inferential analyses. These preliminary analyses indicated good quality data, free from unusual or aberrant observations, on model fit in a Cox proportional hazards regression. The strength of the association between BCKDC, hBCATc and hBCATm was quantified using Pearson's

product moment correlation coefficient for linear association and for monotone association using the nonparametric Spearman's rank correlation coefficient. Both approaches gave the same statistical conclusions. Differences in mean levels for each of BCKDC, hBCATm and hBCATc between IDH+ and IDH- was assessed using the robust independent samples t-test without assuming equality of variances (the Welch-Aspin test) and was triangulated using bootstrapping. Both approaches gave the same statistical conclusions. A two-group Kaplan-Meier analysis of overall survival (OS) using the Mantel Cox log-rank test to judge statistical significance was conducted using a median cut-point on each of BCKDC (median = 0.6), hBCATm (median = 0.40), and hBCATc (median = 0.40). A two-group Kaplan-Meier analysis is robust to the presence of potentially influential observations. In addition, a sensitivity analysis was also performed to ensure conclusions were robust to the choice of cut-point.

RESULTS

All IDH-WT tumours examined have increased expression of the BCAA metabolic proteins relative to normal brain tissue (Hull *et al*., 2012). Western blot analysis has demonstrated that in IDH–WT tumours there was a significant increase in the expression of hBCATm (*p=0.036*) and hBCATc (*p=0.007*) relative to IDH mutation glioblastomas (Figure 1A and B). This increase in hBCAT expression was supported using IHC analysis (Figure 1C). Although several banding patterns for BCKDC are evident in these gliomas, where combinations of 50 and 70 kDa proteins existed, the overall expression between IDH-WT and IDH1 did not show a significant difference (Figure 1A, B and C). Interestingly, substantive expression of BCKDC is evident in IDH1 mutation tumours even though hBCAT expression is largely absent.

Statistically, hBCATm and hBCATc have a high positive correlation coefficient ($r = 0.625$, $p < 0.001$). However, hBCATm is more specific that hBCATc, when considering Overall Survival. A Kaplan-Meier survival curve (Figure 2) and log-rank tests demonstrated that the predominant factor effecting overall survival is IDH status (Figure 2A), with IDH mutation positive gliomas associated with significantly better survival (*p=0.005*). Additionally, for all combinations of IDH-WT gliomas (Grade 2, 3 and 4), those expressing low hBCATm (median split) are associated with better overall survival (Figure 2B *p=0.003*). Moreover, in the Grade 4 only population, and exclusively considering those IDH-WT, a similar Kaplan-Meier curve (Figure 2C) and log-rank analysis shows that low hBCATm is significantly associated with better overall survival (*p=0.043*). The overall summary from these analyses is that hBCATm retains clinical utility across all data. Neither hBCATc nor BCKDC had a statistical effect on patient survival (*p=0.971 and 0.642*, respectively).

Moreover, between tumour grades we observed a significant increase in hBCATm expression (*p=0.010*), indicating that as expression of hBCAT increases prognosis is poorer (Figure 3). Multiple banding patterns for hBCATm are also reported for anaplastic

oligodendroglioma indicating that different isoforms of hBCATm are prominently expressed in these tumour types (Figure 4). In some IDH1 mutation tumours positive hBCATc expression was observed, which was previously considered to be absent in these tumours (data not shown). Also, less expression of the BCKDC upper band at 70 kDa is observed between the stage III AO tumours and the stage IV GBM tumours (observation).

DISCUSSION

Despite aggressive treatment with combined radiochemotherapy and cytoreductive surgery, glioblastomas still have a poor prognosis, highlighting the need for new therapeutic targets and prognostic indicators. However, there is a large degree of variation, with marked differences in survival time attributed to the mutation status of key genetic markers, two of which are IDH and MGMT status (Nikiforova *et al*., 2011). IDH status and methylation of genes is strongly associated with prognosis. Hypermethylation of the MGMT repair enzyme leads to epigenetic gene silencing, preventing the transcription of MGMT, and increasing tumour susceptibility to DNA damage by alkylating agents such as temozolomide, thus improving patient outcome (Esteller *et al*., 2000; Esteller 2000). Improved prognosis is also reported for patients with a mutation in the IDH1 isoform. There are three isoforms of IDH, IDH1 is cytosolic and IDH2 and IDH3 are mitochondrial (Guo *et al*., 2011). IDH1 and IDH2 are $NADP^+$-dependent enzymes that, under normal physiological conditions, catalyse the oxidative carboxylation of isocitrate to α-ketoglutarate as part of the TCA cycle (Reitman *et al*., 2011). In some gliomas, IDH has a point mutation resulting in a single amino acid substitution at arginine-132 in IDH1 and arginine-140 in IDH2 (Gross *et al*., 2010). Both point mutations result in a functional enzyme that produces 2-hydroxyglutarate (2HG) (Gross *et al*., 2010;Dang *et al*., 2010) (Figure 5). Accumulation of 2HG causes methylation of histones within chromatin, preventing uncoiling and can prevent growth by impairment of cellular differentiation (Lu *et al*., 2012). In gliomas with IDH-WT profiles there is frequently an amplification of EGFR (Hartmann *et al*., 2010), which is associated with increased proliferation and radiotherapy resistance (Nakamura 2007). For survival, these tumours have hypermetabolic rates where glucose and glutamine are key metabolic fuels. More recently, an increased expression of hBCATc, a key metabolic protein, was associated with IDH-WT tumours (Tonjes *et al*., 2013). In this study, hBCATc metabolism was shown to contribute to cell proliferation and in IDH1 mutation models, BCAT1 was shown to be hypermethylated resulting in decreased expression, indicating a direct role for hBCATc in tumorgenesis, and a potential therapeutic target (Tonjes *et al*., 2013).

Under normal physiological conditions, the hBCAT isoforms show both tissue and cell specific expression (Hull *et al*., 2012), which makes their co-expression in gliomas most unusual, highlighting new regulatory mechanisms for the BCAT genes under pathogenic conditions. Here, we report that the mitochondrial isoform, hBCATm, was also significantly (*p=0.036*) increased in IDH-WT tumours and more importantly is a negative predictor of patient survival in IDH-WT glioblastomas (*p=0.003*) and also in grade IV tumours alone (*p=0.043*). This is significant because if hBCATc alone was inhibited i.e. the sole BCAA metabolic target, then the activity of hBCATm might hypothetically supersede that of hBCATc attenuating the impact of a targeted therapeutic. As expression of both hBCATs is significantly increased in IDH-WT tumours, we predict that this will generate high concentrations of BCKAs and glutamate, supporting the hypothesis that increased BCKA production contributes to tumour growth and proliferation, dependent on the activity of the BCKDC. Glutamate, also a product of BCAA metabolism, is a precursor for glutamine synthesis, a major nutrient for tumorigenesis. Numerous metabolic studies have supported a BCAA/BCKA shuttle between neuronal and astrocytic cells in rat and cell culture models (Leith *et al*., 2001;Sweatt *et al*., 2004), indicating that in IDH1-WT tumours, a role exists for glutamate and glutamine metabolism, which will be critical for brain neurotransmitter metabolism and detoxification. Increased concentrations of glutamate has already been shown to occur in gliomas, providing multiple benefits to tumour mass, including the increase in the growth rate of tumour cells (Takano *et al*., 2001), and an increased ability to invade the surrounding tissue by increasing cellular motility (Lyons *et al*., 2007). Glutamate release also destroys neuronal cells creating a path for tumour growth (Sontheimer *et al*., 2003). Release of glutamate in exchange for cysteine is controlled by the system x_c^- cysteine glutamine exchanger. The exchanger functions to generate glutathione (GSH) with increased glutamate production leading to a rise in GSH concentration (De Groot *et al*., 2011). In tumours, increased GSH is considered to protect against reactive oxygen species further supporting tumour survival. Therefore, increased hBCAT expression, as reported here, would result in neurological damage by accumulation of glutamate, increased

invasiveness and free radical protection through increased glutamate production, altogether of benefit to tumour survival.

Clearly, expression of hBCATc in IDH-WT tumours presents as a very attractive target for new therapies. However, this new data indicates that with increased hBCATm in IDH-WT tumours one must also consider how their expression will impact tumour growth and survival. Moreover, these studies are the first to illustrate that as the tumour progresses from grade II to grade IV the expression of both isoforms increases, supporting their role as new prognostic markers and indicators of patient survival.

References

1. Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A. (2008) Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol. 116(6):597-602.
2. Conway ME, and Hutson SM. (2015). The Cytosolic and Mitochondrial Branched Chain Aminotransferase. In Branched Chain Amino Acids in Clinical Nutrition (p25-40). Springer New York.
3. Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM. (2010) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 465(7300):966.
4. De Groot J, Sontheimer H (2011) Glutamate and the Biology of Gliomas. Glia: 59 (8), 1181 -1189.
5. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, Herman JG. (2000) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res. 60(9):2368-71.
6. Esteller M. (2000) Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. Eur J Cancer. 36(18):2294-300.
7. Guo C, Pirozzi CJ, Lopez GY, Yan H. (2011) Isocitrate dehydrogenase mutations in gliomas: mechanisms, biomarkers and therapeutic target. Curr Opin Neurol. 24(6):648-52.
8. Gross S, Cairns RA, Minden MD, Driggers EM, Bittinger MA, Jang HG, Sasaki M, Jin S, Schenkein DP, Su SM, Dang L, Fantin VR, Mak TW. Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. J Exp Med. 2010;207(2):339-44.
9. Hartmann C, Hentschel B, Wick W, Capper D, Felsberg J, Simon M, Westphal M, Schackert G, Meyermann R, Pietsch T, Reifenberger G, Weller M, Loeffler M, von Deimling A. (2010) Patients with IDH1 wild type anaplastic astrocytomas exhibit worse prognosis than IDH1-mutated glioblastomas, and IDH1 mutation status accounts for the unfavorable prognostic effect of higher age: implications for classification of gliomas. Acta Neuropathol. 120(6):707-18.
10. Hull J, Patel V, El Hindy M, Lee C, Odeleye E, Hezwani M, Love S, Kehoe P, Chalmers K, Conway M. (2015) Regional Increase in the Expression of the BCAT Proteins in Alzheimer's Disease Brain: Implications in Glutamate Toxicity. J Alzheimers Dis. 45(3):891-905.
11. Hull J, Patel VB, Hutson SM, Conway ME. (2015) New insights into the role of the branched-chain aminotransferase proteins in the human brain. J Neurosci Res. doi: 10.1002/jnr.23558. [Epub ahead of print]
12. Hull J, Hindy ME, Kehoe PG, Chalmers K, Love S, Conway ME. (2012) Distribution of the branched chain aminotransferase proteins in the human brain and their role in glutamate regulation. J Neurochem. 123(6):997-1009..
13. Kreth S, Thon N, Kreth FW. Epigenetics in human gliomas. Cancer Letters -Cancer Epigenetics as Biomarkers of Clinical Significance. 2014;342 (2), p185 -192.
14. Lieth E, LaNoue KF, Berkich DA, Xu B, Ratz M, Taylor C, Hutson SM. (2001) Nitrogen shuttling between neurons and glial cells during glutamate synthesis. J Neurochem. 76(6):1712-23.
15. Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, Edwards CR, Khanin R, Figueroa ME, Melnick A, Wellen KE, O'Rourke DM, Berger SL, Chan TA, Levine RL, Mellinghoff IK, Thompson CB. (2012) IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature. 483(7390):474-8.

16. Lyons SA, Chung WJ, Weaver AK, Ogunrinu T, Sontheimer H. (2007) Autocrine glutamate signaling promotes glioma cell invasion. Cancer Res. 67(19):9463-71.
17. Nakamura J. (2007) The epidermal growth factor receptor in malignant gliomas: pathogenesis and therapeutic implications. Expert Opinion on Therapeutic Targets. 11(4):463-472.
18. Nikiforova MN and Hamilton RL. (2011) Molecular Diagnostics of Gliomas. Archives of Pathology and Laboratory Medicine. 135(5):558 -568.
19. Ohgaki H, Kleihues P. (2013) The definition of primary and secondary glioblastoma. Clin Cancer Res. 19(4):764-72.
20. Pérez-Villaseñor G, Tovar AR, Moranchel AH, Hernández-Pando R, Hutson SM, Torres N. (2005) Mitochondrial branched chain aminotransferase gene expression in AS-30D hepatoma rat cells and during liver regeneration after partial hepatectomy in rat. Life Sci. 78(4):334-9.
21. Reitman ZJ, Jin G, Karoly ED, Spasojevic I, Yang J, Kinzler KW, He Y, Bigner DD, Vogelstein B, Yan H. (2011) Profiling the effects of isocitrate dehydrogenase 1 and 2 mutations on the cellular metabolome. Proc Natl Acad Sci U S A. 108(8):3270-5.
22. Sontheimer H. (2003) Malignant gliomas: perverting glutamate and ion homeostasis for selective advantage. Trends in Neurosci. 26(10)543 -549.
23. Sweatt AJ, Wood M, Suryawan A, Wallin R, Willingham MC, Hutson SM. (2004) Branched-chain amino acid catabolism: unique segregation of pathway enzymes in organ systems and peripheral nerves. Am J Physiol Endocrinol Metab. 286(1):E64-76.
24. Takano T, Lin JH, Arcuino G, Gao Q, Yang J, Nedergaard M. (2001) Glutamate release promotes growth of malignant gliomas. Nat Med. 7(9):1010-5..
25. Tönjes M, Barbus S, Park YJ, Wang W, Schlotter M, Lindroth AM, Pleier SV, Bai AH, Karra D, Piro RM, Felsberg J, Addington A, Lemke D, Weibrecht I, Hovestadt V, Rolli CG, Campos B, Turcan S, Sturm D, Witt H, Chan TA, Herold-Mende C, Kemkemer R, König R, Schmidt K, Hull WE, Pfister SM, Jugold M, Hutson SM, Plass C, Okun JG, Reifenberger G, Lichter P, Radlwimmer B. (2013) BCAT1 promotes cell proliferation through amino acid catabolism in gliomas carrying wild-type IDH1. Nat Med. 19(7):901-8.

Patient number	Age at diagnosis	Diagnosis	Grade	Gender	IDH Status	MGMT status
1	43	Glioblastoma	IV	Male	IDH-	U
2	44	Glioblastoma	IV	Male	IDH-	U
3	61	Glioblastoma	IV	Male	IDH-	U
4	68	Glioblastoma	IV	Male	IDH-	M
5	64	Glioblastoma	IV	Male	IDH+	M
6	46	Glioblastoma	IV	Male	IDH+	M
7	35	Glioblastoma	IV	Male	IDH+	M
8	38	Glioblastoma	IV	Female	IDH+	M
9	26	Anaplastic astrocytoma	III	Female	IDH+	M
10	25	Anaplastic astrocytoma	III	Male	IDH+	U
11	20	Anaplastic astrocytoma	III	Male	IDH+	U
12	58	Anaplastic astrocytoma	III	Male	IDH-	M
13	67	Anaplastic astrocytoma	III	Male	IDH-	U
14	51	Anaplastic astrocytoma	III	Female	IDH-	M
15	61	Oligodendroglioma	II	Male	IDH+	M
16	44	Oligo-Astrocytoma	II	Female	IDH-	U
17	69	Anaplastic Oligodendroglioma	III	Male	IDH-	U
18	54	Anaplastic Oligodendroglioma	III	Male	IDH+	M
19	57	Anaplastic Oligodendroglioma	III	Female	IDH-	M
20	46	Anaplastic Oligodendroglioma	III	Female	IDH-	M
21	46	Anaplastic Oligodendroglioma	III	Female	IDH-	M
22	63	Glioblastoma	IV	Female	IDH-	UK
23	39	Glioblastoma	IV	Male	IDH-	U
24	28	Glioblastoma	IV	Female	IDH+	M
25	32	Oligo-Astrocytoma	II	Female	IDH+	M
26	73	Anaplastic astrocytoma	II	Male	IDH-	M
27	44	Anaplastic astrocytoma	III	Male	IDH-	U
28	54	Glioblastoma	IV	Female	IDH-	U
29	47	Glioblastoma	IV	Male	IDH-	M
30	68	Glioblastoma	IV	Male	IDH-	M
31	72	Glioblastoma	IV	Female	IDH-	M
32	56	Glioblastoma	IV	Male	IDH-	U
33	67	Anaplastic Astrocytoma	III	Male	IDH-	U
34	66	Glioblastoma	IV	Male	IDH-	M
35	51	Glioblastoma	IV	Male	IDH-	M
36	43	Glioblastoma	IV	Female	IDH-	M
37	71	Glioblastoma	IV	Female	IDH-	M
38	55	Glioblastoma	IV	Female	UK	M
39	68	Glioblastoma	IV	Male	IDH-	U
40	64	Glioblastoma	IV	Female	IDH-	M
41	66	Glioblastoma	IV	Female	IDH-	M
42	48	Glioblastoma	IV	Male	IDH-	U
43	61	Glioblastoma	IV	Male	IDH-	U
44	58	Glioblastoma	IV	Female	IDH-	U
45	57	Glioblastoma	IV	Female	IDH-	M
46	65	Glioblastoma	IV	Female	IDH-	M
47	52	Glioblastoma	IV	Male	IDH-	M
48	74	Glioblastoma	IV	Female	IDH-	U
49	53	Glioblastoma	IV	Male	IDH-	U
50	58	Glioblastoma	IV	Male	IDH-	M
51	34	Oligo-Astrocytoma	II	Male	IDH+	M
52	24	Oligo-Astrocytoma	II	Female	IDH+	M
53	29	Oligo-Astrocytoma	II	Female	IDH+	M
54	39	Diffuse Astrocytoma	II	Female	IDH+	M
55	49	Oligo-Astrocytoma	II	Female	IDH+	M
56	UK	Diffuse Astrocytoma	II	UK	IDH+	M
57	UK	Diffuse Astrocytoma	II	UK	IDH+	M
58	50	Oligo-Astrocytoma	II	Female	IDH+	M

Table 1: Patient details, IDH/MGMT of sample Glioblastomas

FIGURE LEGENDS

Figure. 1. Increased hBCAT expression in IDH-WT gliomas relative to IDH1 tumours. Western blot analysis and immunohistochemistry was used to evaluate the expression of BCKDC, hBCATm and hBCATc in IDH-WT and IDH1 mutation gliomas. **Panel A**: Patient details and Western blot analysis of glioma homogenates. The density of each band was measured using ImageJ™ software and analysed for significance using a one-way ANOVA test in Minitab™ (n=55). **Panel B:** Panels show interquartile range (box) sample variability (whiskers) and the median (horizontal line within the interquartile range) of Western blot analysis for BCKDC, hBCATm and hBCATc relative to α-tubulin. Statistical analysis reveals a significant increase in hBCATm (*p=0.036*) and hBCATc (*p=0.007*) protein expression in IDH-WT gliomas compared to IDH mutation gliomas. **Panel C:** BCKDC, hBCATm, hBCATc staining of glioma sections, where increased staining was observed for hBCATm and hBCATc for IDH-WT relative to IDH mutation gliomas (n=51). Magnification for all sections, X20. Abbreviations: F – female; M – male; Me – methylated; MGMT – methylguanine-DNA methytransferase methylation; U – unmethylated.

Figure. 2. Kaplan Meier analysis for IDH status and hBCATm expression. Statistics were calculated from Log rank Mantel-Cox analysis. **Panel A:** Cumulative survival of IDH status demonstrates significantly increased rate of survival in IDH mutation gliomas compared to IDH-WT (p=0.005) (n=47). **Panel B:** Cumulative survival of hBCATm expression in IDH-WT patients (including grades 2, 3 and 4): demonstrates significantly increased rate of survival in low (median split) hBCATm expressing gliomas (p=0.003) (n=35). **Panel C:** Cumulative survival of hBCATm expression in IDH-WT patients (grade 4 only) demonstrates significantly increased rate of survival in low (median split) hBCATm expressing gliomas (p=0.043) (n=24).

Figure. 3. hBCAT expression increases with tumour grade. Western blot analysis of BCKDC, hBCATm and hBCATc in glioma samples, grade 2-4. There is a significant increase in hBCATm (*p=0.010*) with increasing grade, where a trend for increased hBCATc is also observed (n=55). Abbreviations: F – female; M – male; Me – methylated; MGMT – methylguanine-DNA methytransferase methylation; O1 – Oligodendroglioma; O2 – Oligo-Astrocytoma; U – unmethylated.

Figure. 4. Multiple banding for hBCATm in Oligodendric gliomas and Anaplastic oligodendrogliomas. Western blot analysis of BCKDC, hBCATm and hBCATc in IDH-WT and IDH mutation glioma samples. Abbreviations: F – female; M – male; Me – methylated; MGMT – methylguanine-DNA methytransferase methylation; O1 – Oligodendroglioma; O2 – Oligo-Astrocytoma; U – unmethylated.

Figure 5. IDH-WT and IDH1 mutation metabolism of citrate, linked with BCAA metabolism. A. IDH-WT catalyses the oxidative carboxylation of isocitrate to α-ketoglutarate as part of the TCA cycle. **B.** In glioblastomas with IDH mutation the product of catalysis is 2-hydroxyglutarate, accumulation of which causes methylation of histones within DNA, ultimately impairing differentiation. **C.** In IDH-WT tumours increased hBCAT expression contributes to tumour survival and neurotoxicity of neighbouring healthy brain cells.

Table 1. Glioma cases used in immunohistochemical and Western blot analysis of BCKD, hBCATc and hBCATm. All cases were from the South West Brain Tumour Bank (Southmead Hospital) and had no significant comorbidities (such as Vascular dementia or Parkinson's disease). Abbreviations: M – methylated; MGMT – methylguanine-DNA methytransferase methylation; U – unmethylated; UK - unknown.

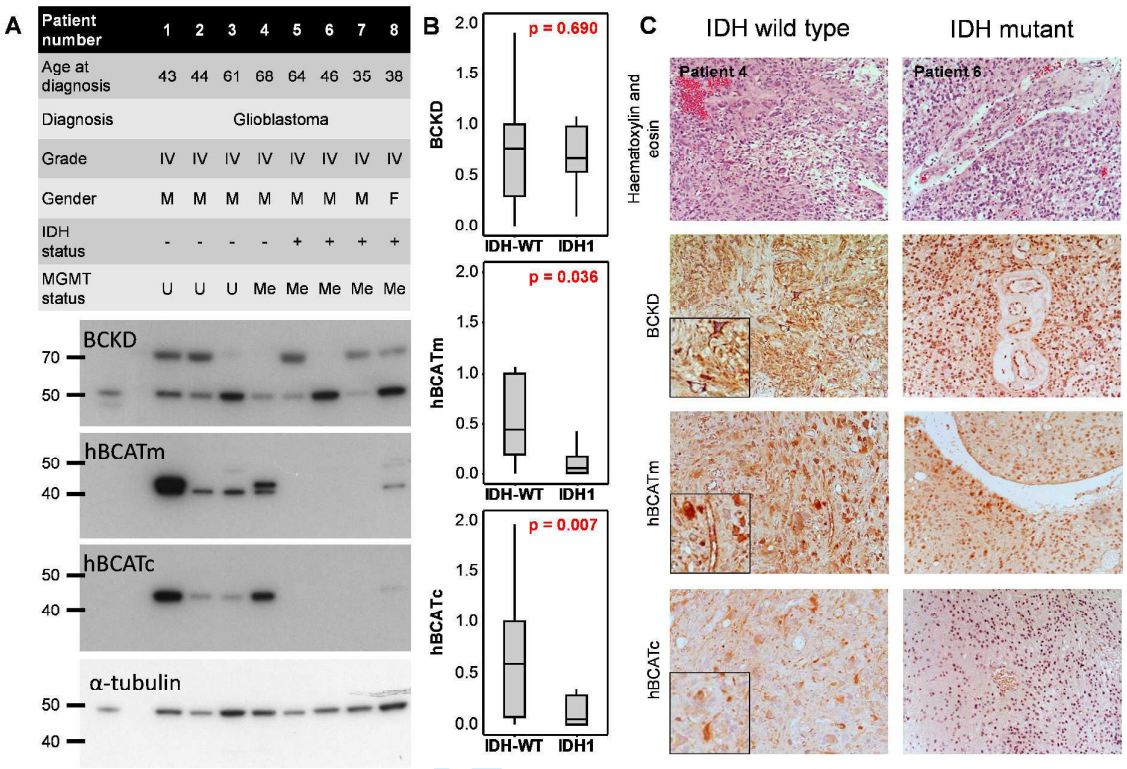


Figure 1

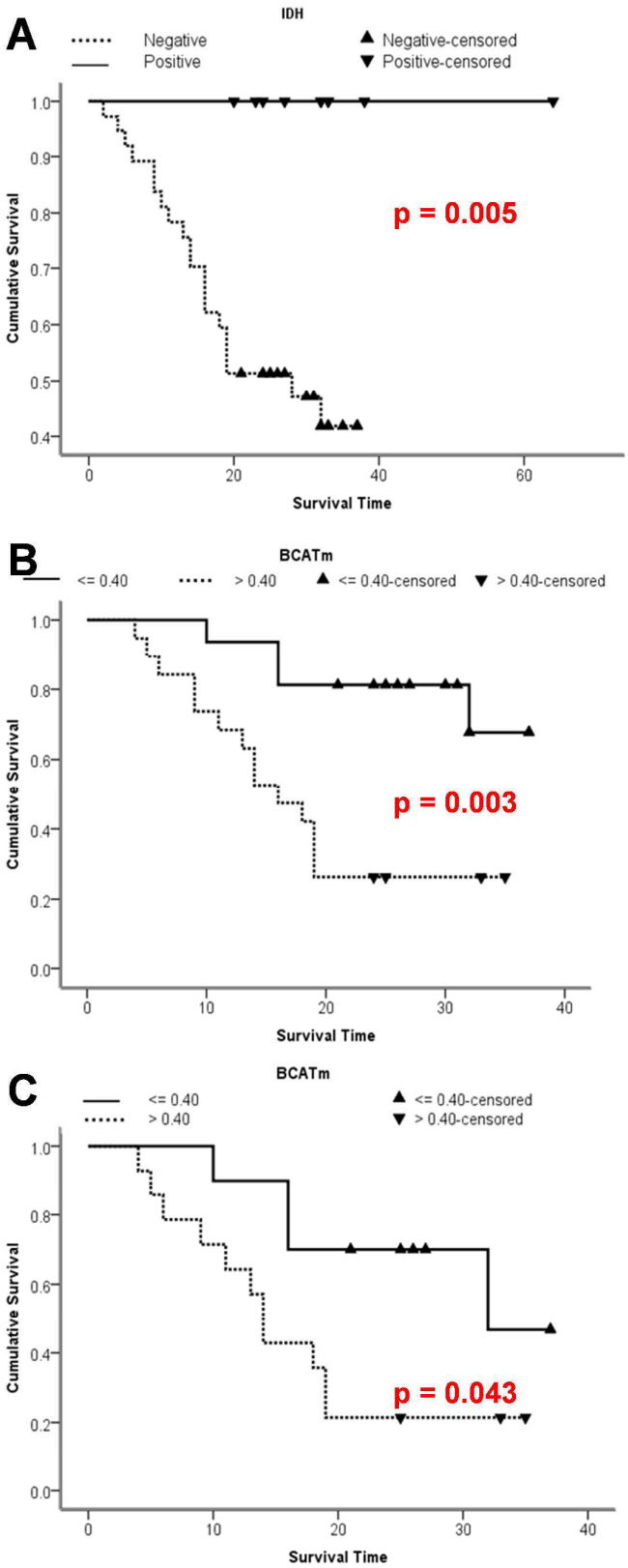
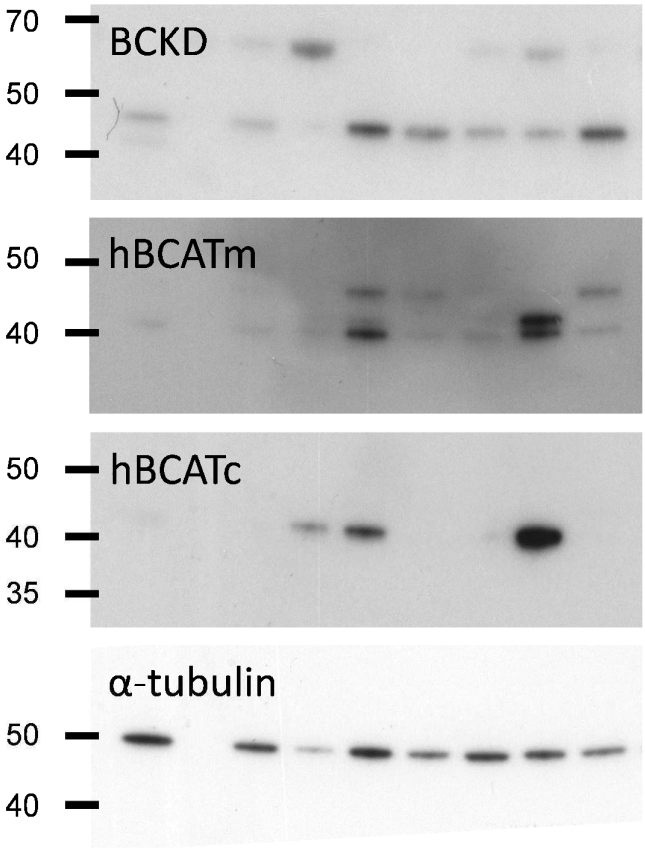

A
IDH
Negative
Positive
Negative-censored
Positive-censored
Cumulative Survival
p = 0.005
Survival Time
B
BCATm
<= 0.40
> 0.40
<= 0.40-censored
> 0.40-censored
Cumulative Survival
p = 0.003
Survival Time
C
BCATm
<= 0.40
> 0.40
<= 0.40-censored
> 0.40-censored
Cumulative Survival
p = 0.043
Survival Time

Figure 2

A

Patient number	15	16	13	11	10	1	6
Age at diagnosis	61	44	67	20	25	43	46
Diagnosis	O1	O2	Anaplastic astrocytoma			GM	
Grade	II	II	III	III	III	IV	IV
Gender	M	F	M	M	M	M	M
IDH status	+	-	-	+	+	-	+
MGMT status	Me	U	U	U	U	U	Me



B

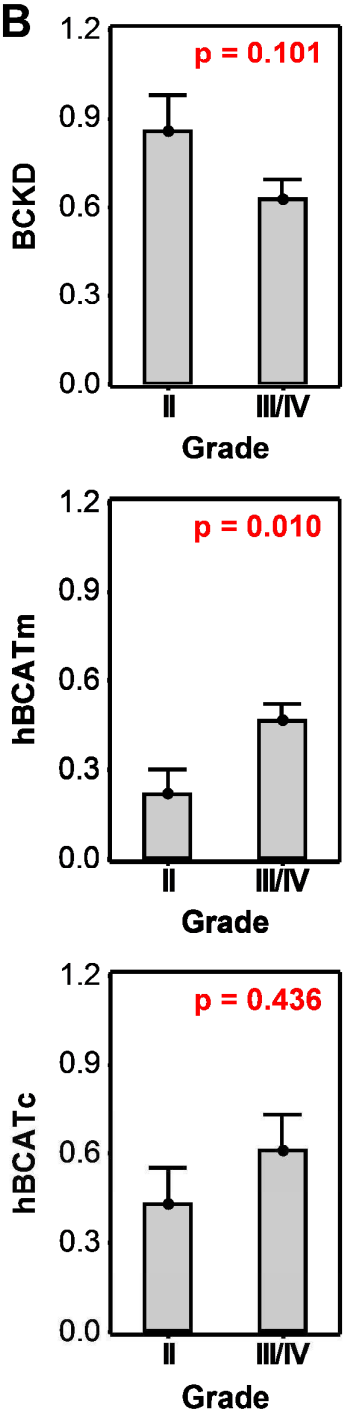


Figure 3

Patient number	15	16	17	18	19	20	21
Age at diagnosis	61	44	69	54	57	46	46
Diagnosis	O1	O2	Anaplastic oligodendrogioma				
Grade	II	II	III	III	III	III	III
Gender	M	F	M	M	F	F	F
IDH status	+	-	-	+	-	-	-
MGMT status	Me	U	U	Me	Me	Me	Me

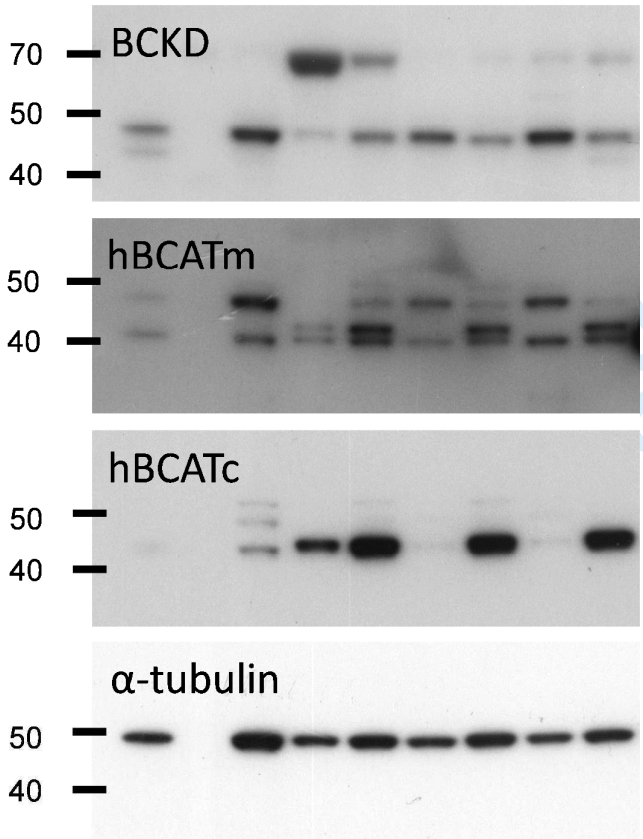


Figure 4

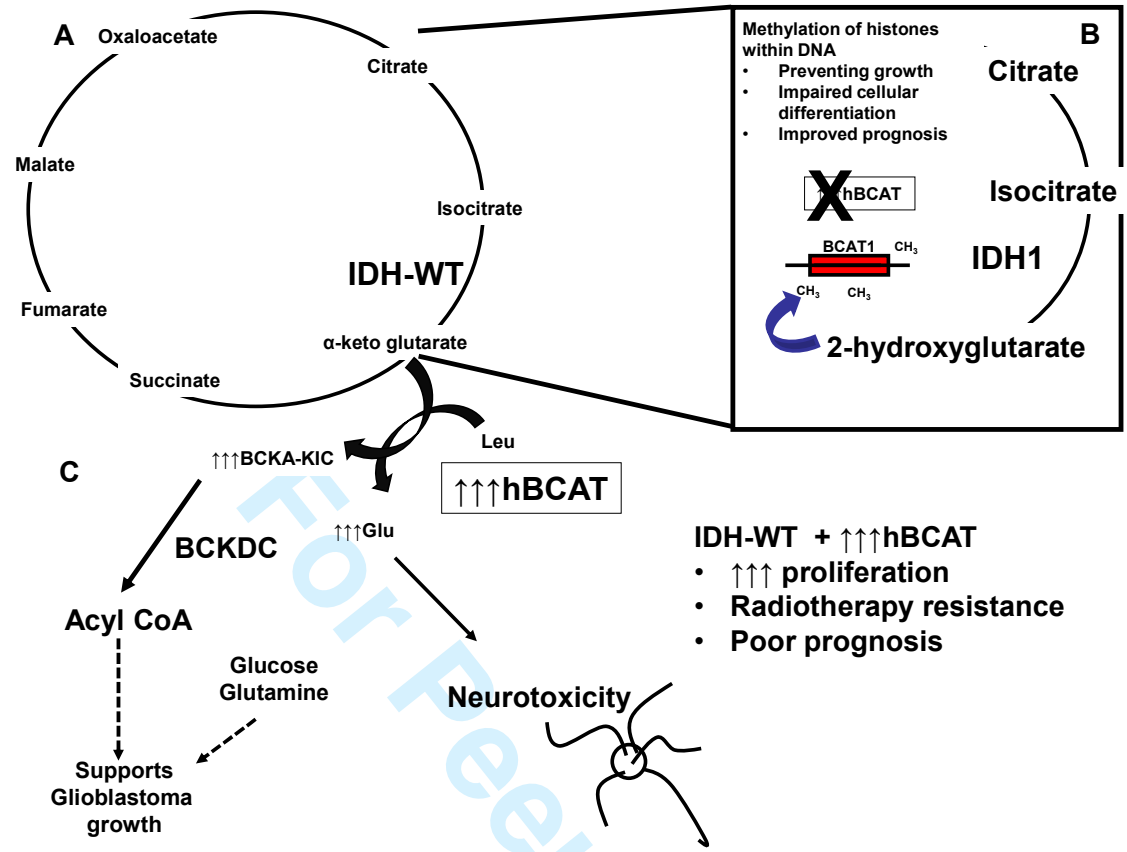


Figure 5